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The Nitrogen Complexes of the Heart Phosphatides

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THE NITROGEN COMPLEXES OF THE HEART PHOSPHATIDES

BY

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

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DEGREE OF MASTER OF ARTS.

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THE NITROGEN COMPLEXES OF THE HEART PHOSPHATIDES.

TABLE OF CONTENTS.

I. GENERAL STATEMENT.

Investigations on the influences of substances present within animal tissues on the life of the animal are being carried out more and more. Studies of the tissues themselves, of the processes of metabolism within them and of the interdependence as well as the independence of the important food stuffs are continually reported. Data for knowledge of conditions governing these actions are secured from researches on the physiology and chemistry of the interacting substances. For the physiology the data are taken from experiments on living animals. Conclusions drawn from these experiments enable the investigator to discuss the physiological aspect of his problem. For the chemistry the method of investigation is somewhat different. Here the interacting substances must be studied as actual chemical compounds; their structure, properties and behavior as chemical units and as constituents of a mixture must be determined. From data thus secured they can be placed in their position in the great groups of chemically or physiologico-chemically important substances.

Such a group is the one called lipoids or lipines. The lipoids are defined as the fat-like constituents of the cells which can be extracted by ether and similar organic solvents. The distinction between fats, which are also taken up by these solvents, and lipoids is that the fats are fatty acid esters of glycerol, while the lipoids are of a more complex nature. The greater complexity of the lipoids is shown by the classifi-

cation. According to the scheme of Rosenheim[1] lipoids are divided into-

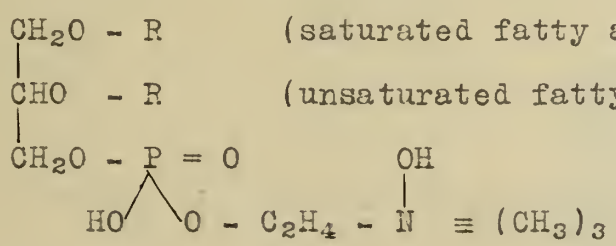
(1) Cholesterin group, free from nitrogen and phosphorous

(2) Cerebrogalactosides, contain nitrogen but no phosphorous

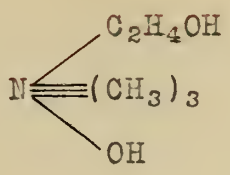
(3) Phosphatides, contain both nitrogen and phosphorous.

In this work only the latter group will be taken up. The phosphatides or phospholipines are defined in text books[2] of physiological chemistry as "the fatty acid esters of glycerophosphoric acid combined with a nitrogenous base, usually choline." The acids in the molecule are the higher members of the saturated and unsaturated series. Palmitic acid, $C_{15}H_{31}COOH$ and stearic acid, $C_{17}H_{35}COOH$ are the saturated acids usually found; while oleic acid $C_{17}H_{33}COOH$ and also members of the more unsaturated series, i.e. the linoleic and linolenic series are reported.

Lecithin, which is found throughout the body, is the compound most frequently given as an example of the phosphatides, its formula being represented as-



On hydrolysis lecithin was supposed to break up into the fatty acids; glycerol and phosphoric acid, or as is usually the case glycerophosphoric acid, and choline. Choline is oxyethyl trimethyl ammonium hydroxide



It has been known for some time that all the nitrogen present in lecithin is not in the form of choline. It was with a view of working up the nitrogen complexes of heart lecithin and of another phosphatide in beef hearts, cuorin, that this investigation was started.

The phosphatides are further subdivided by Rosenheim on the basis of the ratio of nitrogen to phosphorous within the molecule. Lecithin, described above, has a nitrogen phosphorous ratio of one to one. It is therefore a mono-amino monophosphatide. Cuorin, the characteristic phosphatide of the heart, is a mono-amino diphosphatide (N:P = 1:2). Another substance found in the heart muscle is a diamino monophosphatide (N:P = 2:1). Triamino monophosphatides and triamino diphosphatides have also been isolated from egg yolk and ox kidneys. The classification also serves as a criterion of the purity of the phosphatides secured on extraction, since in rather pure substances the nitrogen-phosphorous ratio is simple.

The physiological importance of the phosphatides is not definitely known. This is due to the uncertainty as to what phosphatides really are, whether substances hitherto isolated are pure compounds or mixtures incapable of being separated by methods now in use. Their general place in the group of lipoids is known but their chemical structure, properties and relations to other substances have not been worked out. When they are

definitely characterized, then a thorough explanation of their value to the body may be evolved.

The general distribution of phosphatides throughout the body would show of itself that they are of importance for the maintenance of normal conditions. They make up twenty to twenty-five per cent of the solids of brain and nerve tissue. As a result much of the investigation on phosphatides has been made on material secured there. Egg yolk, embryonic tissue, contains nine per cent of its dry weight as phosphatides. Heart, liver, lungs, kidney, pancreas, voluntary and involuntary muscle are other tissues whose phosphatides have been studied.

Ideas have been entertained that phosphatides may serve as a transitional stage in the break-down of proteins into fat, thus giving them a direct nutritional value. Work has also been reported by Stepp[3] in which he speaks of their absolute necessity for the diet. This investigator gave to mice food which had been extracted thoroughly with alcohol and ether. In a short time the mice died. After being fed for a few weeks with extracted material some were put on a diet containing the residue of the alcohol-ether extracts of egg yolk along with the extracted food. The extract had been concentrated at a low temperature in order to prevent decomposition of the substances. While on the treated food the mice had become weak, they soon came to their normal condition after the addition of the lipoid containing extract. Stepp eliminated the possibility of decline in health due to absence of fats and salts by supplying these in normal proportions. Only

the phosphatides were omitted.

Of all authors Ivar Bang[4] makes the most extensive claims for the necessity of lipoids, particularly phosphatides. He discards the idea that proteins are the chief cell constituents, saying that it has not been proved that proteins in themselves are absolutely essential to life. He calls the lipoids or more particularly combinations of lipoids with proteins, the important substances. On account of their ease of change within the cell they are considered the true labile substance of protoplasm. In his treatment he does not deal with lipoids as direct agents in the cell metabolism but more as physical factors. The lipoid membrane, composed chiefly of lecithin, around each cell serves in many ways, regulating by changes in permeability the intracellular metabolism and the influence of foreign substances such as narcotics and hemolyzing agents.

Koch[5] states that the direct significance of the phosphatides is two-fold. In colloidal solutions phosphatides and proteins maintain the viscosity of the cell contents, the lability of the former towards salts being the regulating factor. In metabolic activities they are concerned with the oxygen interchange on account of the presence of the unsaturated acids and are perhaps connected by means of their nitrogen containing radical to nitrogen metabolism.

The theories held by investigators as to the physiological value of the phosphatides are thus seen to differ greatly. It is certain that the properties described can be

attributed in part at least to the phosphatides. When the importance of these properties to the body is considered, one wonders that more investigations have not been made, as a review of the literature does not show nearly the number of contributions that other less important subjects have called forth. In recent years, however, the problem has been taken up from physiological and chemical standpoints and advances in the knowledge of the properties and functions of phosphatides may be expected.

II. HISTORICAL.

The phosphatides of the heart are closely related to those found in other tissues so that results of any investigations of the problem may be applied to a study of these compounds in the heart muscle. The position reached by investigation of the phosphatides is most easily shown by reference to a few of the important articles on the subject.

The phosphorized fats or phosphatides have been investigated intermittently for a great many years. Among early researches the study of egg lecithin and its hydrolysis products by Hoppe-Seyler and his pupil Diakonow was of value as a basis for future work. For separation of the phosphatides after extraction from the tissue, Zuelzer, working with brain and nerve tissue, devised the acetone and alcohol precipitations now in use.

The foundation of more modern work on phosphatides was laid by Thudichum[6]. This investigator, also working on brain tissue, made separations by virtue of the insolubility

in organic reagents of certain metallic derivatives of the phosphatides. Separation was made more complete by differences in solubility of the phosphatides themselves, after they had been freed from combination with the metals. In general the plan devised by Thudichum is carried out today. He also identified some of the hydrolysis products of the material secured from brain.

Erlandsen[7] carried out a very thorough research on phosphatides of the heart, their preparation and properties. Frequent reference will be made to the result of this investigation. The article is chiefly descriptive, giving methods of preparation and analysis, showing similarities between substances secured from ether and alcohol extracts and dealing with the cleavage products of the preparations. Erlandsen characterized the two chief phosphatides, lecithin and cuorin, to a high degree of accuracy but did not deal extensively with the nitrogen complexes. He reported that in cuorin the base is not choline since on hydrolysis with barium hydroxide a precipitate was secured with platinum chloride which yielded a different amount of platinum (37.26%Pt) than that found in the choline compound (31.64% Pt). In lecithin he reported an inability to secure a calculated yield of the platinum chloride salt of choline (42% of theory).

Fränkel[8] made a thorough investigation of the phosphatides of the brain using various extractive treatments. Of interest is his report of Thudichum's statement that on hydrolysis of kephalin, the characteristic phosphatide of the brain,

choline and two other bases were found, and the suggestion that these latter might be oxydimethyl-amine and oxyethyl-amine. He also reported the work of Koch on kephalin, wherein on hydrolysis a substance was found whose platinum salt seemed to correspond to that of monomethyl-oxyethyl amine.

An investigation which bears more directly on the nitrogen containing radicals of the phosphatides is that of MacLean. Lecithin was prepared from egg yolk and ox hearts and hydrolized, with a view of ascertaining the causes of the losses in nitrogen produced by the hydrolysis. Both dilute acids and alkalies were used for hydrolysis but relatively no difference was found in the choline nitrogen. MacLean found the greater part of the nitrogen in heart lecithin to be present in the filtrate from the platinum chloride precipitation. This fraction was not so large with egg lecithin . There is also a small amount that is apparently unhydrolizable, which is reported as being in the fatty acid residue.

His results are given in percentages of total nitrogen present.

	Choline N.	Residue N.
Heart lecithin	41.4	5.34 - 8.48
Trade "	77 - 79	8.5
Egg "	63 - 66	Not given.

The variations in these results lead him to these conclusions: (1) lecithins in different tissues are different, (2) the nitrogen of lecithin is not all present as choline, (3) the formula usually given for lecithin is not correct.

MacLean continued his work on the phosphatides using material secured from horse kidneys for a descriptive investigation[10]. He showed that these compounds in the kidneys are similar in general properties to those in other tissues. Besides lecithin a phosphatide closely resembling cuorin was found. He did not take up the nature of the nitrogenous complexes of the preparations. A description was given of "carnaubon" an ether insoluble phosphatide isolated from the ethereal and alcoholic extracts. In connection with his work on the substances of the kidney MacLean brought out a method for their purification by means of emulsification with water and precipitation of the emulsion with acetone.

For the further study of nitrogen distribution on hydrolysis of phosphatides a method reported by Neuberg and Kerb for precipitation of mono-amino acids has proved useful.[11] This treatment consists of precipitating the acid by addition of mercuric acetate to the solution which is kept alkaline by sodium carbonate. The acid is precipitated as a mercurio-mercuric oxide compound. The precipitate may be broken up by passing H_2S through a water suspension and the hydrochloride of the acid secured by evaporation of the filtrate.

A study of the hydrolysis products of kephalin has been made in this laboratory, and a mono-amino acid isolated by means of the above method. Ammonia has also been found in the alcohol insoluble residue from the hydrolysis solution.

This brings the study of the phosphatides of the heart and related tissues down to the present time. Discussion

of the results of the different investigations will be taken up in the discussion of the data secured in the experimental work outlined on the following pages.

III. EXPERIMENTAL.

The experimental work here reported can be divided into two sections, (A) preparation of material together with various side issues that came up during this process; (B) hydrolysis of the compounds and attempt at identification of some of the hydrolysis products.

A. Preparation.

For the general preparation the method given by Erlandsen was followed, although at different stages digressions were made from it.

Beef hearts weighing 1800 to 2500 grams in the fresh state were used as a source of the material for this experiment. After removing the adherent fat, the valves and the connective tissue as completely as possible, the muscle was minced by passage through a grinder. The ground meat was pressed to remove the blood, weighed approximately and dried. Drying was accomplished by one of three methods, (1) dehydration with alcohol, (2) dehydration with acetone, (3) removal of water by current of air in a drier at a temperature of 35-40°C.

In the dehydration by alcohol the ground material was placed in large bottles and treated with twice its volume of 95% alcohol, being shaken frequently. The first treatment with alcohol extended 12-16 hours at room temperature. The

liquid was then removed by filtration and pressure and fresh alcohol added. The alcohol was allowed to stand on the tissue for two or three days, the bottle being shaken often during this time. At the end of this period all water was considered as taken up by the alcohol so the tissue was removed, dried in a current of air and either placed in a desiccator or treated immediately for extraction of the phosphatides.

After dehydration with alcohol the tissue was of a stringy fibrous nature and apparently quite porous. To give greater surface for extraction it was passed through a coffee mill, but on account of its fibrous condition did not powder well. The treatment with alcohol removes roughly seventy-seven per cent of the original weight of the material as water. Erlandsen advises strongly against the use of alcohol as a dehydrating medium on account of what he calls the danger of oxidation of unsaturated acids in the phosphatide molecule. In this work no especial difference was noted in the character of the extracts from heart muscle dehydrated by alcohol and by other methods.

The alcohol-water solutions were strongly colored, having dissolved tissue pigments, extractives and some phosphatides. In the first dehydration practically no phosphatides are present. In the second, however, it was noted that on treatment to get the substances in solution, a good yield was obtained. This seems to be the characteristic action noted in dehydration by alcohol. The solubility of the substances in the ether used later is also affected by this treatment, for in the case of the alcohol dried hearts the acetone precipitate (see below) from the ether extract was in every case small in proportion

to the weight of material employed. However, the amount from both alcohol and ether totalled in proportion to that secured by extraction of tissue dried by other methods.

Water was removed from one lot of tissue by acetone. The procedure was the same as when alcohol was used. By this treatment a loss of water corresponding to eighty per cent of the original weight of the tissue was observed. The tissue after the dehydration was light brown and granular, in excellent shape for extraction. Both acetone-water solutions were colored a yellow-brown, the color in the second being stronger than that of a similar alcoholic dehydration of another lot of tissue. On concentration of the liquids and treatment for phosphatides the acetone precipitate was too small to work with. The concentrated solutions gave off an odor much resembling that of beef extract. Therefore it is probable that the acetone dehydration is without solvent action on the phosphatides of the heart, only the extractives and pigments being removed with the water.

Dehydration by means of a current of warm air was the method used for the preliminary preparation of the greater part of the tissue. The finely divided fresh material was spread in thin layers on large glass plates and set in the drying apparatus. This consisted of a long wood chamber containing racks for fifteen plates, fitted with an electric heater at one end and a fan at the other. By adjusting the amount of current in the heating coils and the speed of the fan the temperature within the drier could be regulated at will. A fairly rapid current

of air at 35°-40°C was used. The tissue was treated in this way for 8 to 12 hours by which time it had thoroughly dried on top and partly through the layers. It was then turned over on the plates and allowed to remain in the drier 5-12 hours more. At the end of this period the tissue was dark brown, hard and brittle. It was removed from the plates, ground up somewhat in a mortar and put through a powdering mill. After this treatment it was a fine light colored powder, slightly sticky to the touch. It gradually became darker on exposure to the air.

This system removes only the water from the tissue. The average from several lots showed that approximately seventy-seven per cent of the fresh material is water.

After dehydration comes extraction of the phosphatides and other substances soluble in organic reagents. For the primary extractions ether was the solvent employed, though petroleum ether was tried out with one portion. Ether treatments used were (1) cold extraction in flasks along with the tissue, (2) cold extraction in a modified Soxhlet apparatus,and (3) extraction with the warm solvent. These processes were not carried on simultaneously with different portions of tissue but were taken through in separate stages so that at one time there might be carried on several treatments for dehydration, extraction and purification.

In the first method portions of dry tissue amounting to 150-300 grams were placed in large Erlenmeyer flasks and one liter of ether added. The flasks and contents were set away in the dark, but were shaken up frequently. The extraction proceeded at room temperature for periods ranging from five days

to two weeks.

After the removal of the liquid the tissue was treated with fresh ether and allowed to stand again for several days. One lot of tissue received three of these cold ether treatments.

The extracts secured in this way were clear liquids ranging in color from light yellow to orange brown depending on the amount of dissolved substances. Extracts from air dried tissue were always darker than those from tissue dried by other methods, though no larger amount of phosphatides was found. This difference is due to the fact that in the former the fats, pigments and extractives were still present up to the ether treatment while in the latter they had been removed in great part by the alcohol and acetone.

Extraction by means of the modified Soxhlet apparatus designed in this laboratory, for use with large quantities of tissue, was carried out as follows: 1200-1400 grams of dried tissue were placed in a cage within the copper extractor used. This tissue was treated by conducting ether vapors from a flask into the condenser above the extractor. The condensed liquid dropped on the tissue, was distributed through it by means of cloths, and passed out of the cage carrying with it the soluble substances. When the ether in the extractor reached a height corresponding to that of a siphon tube on the exterior it automatically passed back into the flask. Vaporization and cooling of the solvent before it reached the tissue admitted of the use of distilled products, for the flasks could be changed just before the extract siphoned over, thus keeping it free from contamination by non-volatile bodies in the ether. Also the

treatment was carried out in a vapor of ether, thus preventing oxidation of the unstable substances.

This process also served as a means of concentrating the ether extract. The ether was boiled off from the solution and used for a second treatment without disconnecting the apparatus. When the extract became quite concentrated the flasks were changed and the concentrated portion worked up as described below. The liquid from the second treatment went through the same process so that the same ether, with some additions, was used several times. It was found that three of these treatments removed all substances obtainable in the primary extracts. The third solution contained only a little phosphatide material.

The solutions secured in this way are similar to those from the cold extracts but contained more dissolved substance. They were not always clear, due to small particles of tissue being carried over, but the impurities were removed in the succeeding stages of preparation of the phosphatides.

For the treatment with warm ether the solvent was placed directly in the copper extractor. On warming, the vapors passed up around the cage containing the tissue to the condenser. The warm condensed ether then passed through the tissue as in the other treatment. The characteristic feature of this method is that the tissue is completely surrounded and permeated by ether vapor, the extraction going on at 34°.

This system of extraction is thorough. The first treatment yielded almost all the ether soluble substance. The solution was never quite clear, filtration being necessary to remove the suspended material. When the hot extraction alone

was used two treatments were sufficient. Two lots of the material previously extracted twice by the first (cold ether in flasks) method gave a good yield with the first hot extraction and only a slight one on re-treatment.

With two small portions of tissue the primary extracts were made with petroleum ether. Petroleum ether labelled as 25°-40° fraction was separated into (A) a 25°-40° and (B) a 40°-60° fraction and each added to 160 grams of tissue. The treatment was the same as in the cold ether extraction.

The solutions, after removal from the tissue, appeared to be fairly concentrated. The yield of acetone precipitate from "A" compared well with that from ether extractions. The actual difference between it and the one from "B" was slight but the lower boiling fraction was easier to work with. Second treatments gave practically no phosphatides. The tissue became red during the course of the extracting and this color persisted even after the solvent had been driven off.

After the primary ether extractions were completed boiling alcohol was used. The method in this treatment was the same as that for warm ether. For 1200-1300 grams of tissue two liters of 95% alcohol were taken.

After one extraction by alcohol lasting 13-16 hours, the liquid was siphoned off. The first alcohol extracts were brown red, rather viscous liquids with a pungent odor. Another extraction with warm ether for 8-10 hours was now made to see if the hot alcohol had broken up any phosphatide combination within the tissue, releasing more ether soluble substances. Some phosphatide was found. After the last ether fraction

had been removed the material was subjected to two more alcoholic extractions. The solutions from these were dark brown liquids, very concentrated but not containing much phosphatide.

This completed the extraction of the tissue. The substances in solution were secured by concentration of the extracts. The liquids were distilled in vapor of CO_2 or ether in order to lessen the chance for oxidation. Since heat aids the oxidation the temperatures were kept low. The extracts with ether were distilled at atmospheric pressure; those with higher boiling solvents, in a partial vacuum. Evaporation was continued until the residue attained a syrupy consistency.

These concentrates were taken up in anhydrous ether, prepared by distillation over magnesium bromide. At this point there was always some insoluble material which Erlandsen calls residue A. It is a grayish white granular substance insoluble in ether, forming a fine suspension in it. Erlandsen reported it as consisting principally of inorganic salts, but said that the amount of substance secured was small. In this experiment a fair yield was found in all cases. Also the substance did not seem to consist so much of inorganic salts. It was separated by allowing the ether solution to stand in the refrigerator when the insoluble portion settled out or by centrifuging. The former method was the more satisfactory, for evaporation during centrifuging tended to remove other substances from the solution. A small amount of the compound shaken with an excess of anhydrous ether colored the liquid only slightly. This coloration is probably due to previous incomplete separation of soluble material. However, this liquid gave no precipitate when treated

with acetone or alcohol.

MacLean reported the isolation from the kidneys by similar treatment, of a substance probably a diamino-monophosphatide. His compound was present in a very small proportion, .55 g being secured from 2075 g dry tissue. In this experiment a little over 2 g were found in 1310 g dry tissue. This is the amount present after the inorganic salts had been removed from the insoluble material.

Analysis showed these proportions:

Nitrogen determination by Kjeldahl method.

.5090 g required 8.83 cc. N/10 H_2SO_4

$\%N = 2.43$

The concentrates from the alcoholic extracts behaved in various ways on treatment with anhydrous ether. One portion of tissue gave practically no ether insoluble material in the first alcoholic extract. Others gave rather large amounts, consisting partly of a heavy oil and partly of gray granular matter. The second and third extractions showed decreasing quantities of ether soluble material, the third extract rarely containing phosphatides. The ether insoluble substances from these extracts were not uniform in appearance or in nitrogen content. They all possessed a distinct odor, resembling that of beef extract.

To the clear ether solutions secured from these separations of insoluble material three to four volumes of anhydrous acetone were added to precipitate the phosphatides. In some cases a whitish flocky precipitate formed at once, and always a brown waxy one settled out in a few minutes. The

acetone-ether mixture was placed in a desiccator in a vapor of CO_2 and allowed to stand at least ten hours. On standing, a fat-like substance crystallized out and settled on the precipitate and on the walls of the beaker. This is probably not phosphatide. It is difficult to separate it mechanically from the phosphatide precipitate.

In the preparations from tissue dried by alcohol practically all the acetone precipitate from ether extractions was of the flocky or else of the granular type. The waxy portion appeared as the precipitate secured by treatment of the alcoholic dehydrations.

The acetone-ether solutions were filtered off, the precipitates dried and weighed roughly. This acetone precipitate was the only means used of determining extent of extraction. The weights given are of comparative value only since in both cases the preparations did not consist solely of lecithin and cuorin.

Dry Tissue.	Acetone Precipitates from Different Extracts.				
Weights in grams	1st ether,	2nd ether,	1st alcohol,	3d ether,	2nd alcohol
1170	34	10	40	3	6

For comparison a table from Erlandsen's article and results secured here are shown:

	No. of hearts	Dry tissue	Acetone Precipitates Ether Extract.	Alcohol Extract
Erlandsen's results	8	1440 g	52 g	96 g
This work	6	1170 g	48 g	46 g

In these experiments the great increase with alco-

holic extraction reported by Erlandsen was not observed.

The acetone precipitates were thoroughly dried, then treated again with anhydrous ether. On this treatment there was in every case much undissolved substance. This seemed to be the same as that found on treating the concentrated ether extracts. Apparently the acetone broke up a combination between the insoluble substances and the phosphatides, releasing the former. The ether solution was precipitated again by acetone, this precipitate being used for the treatment for separation of cuorin and lecithin.

The acetone ether solutions varied in color from a pale yellow to a deep red brown. Treatment of test portions with H_2PtCl_6 gave a precipitate, showing phosphatides to be present in small amounts. Fats and extractives are also in the solution. On evaporation a sticky fat-like mass remained. A portion of the mass treated with ether dissolved completely but gave no precipitate immediately with an excess of acetone. A slight turbidity which, after long standing, settled out as a brown waxy substance was observed.

For the separation of cuorin and lecithin the second acetone precipitates were treated again with ether. In every case practically all the material dissolved, forming clear or slightly opalescent dark red or brown solutions.

Three or four volumes of absolute alcohol were added to each ether solution to secure cuorin, the alcohol insoluble phosphatide. The first alcoholic treatments gave two forms of precipitate, a brown waxy substance settling out immediately and a white flocky portion that separated out after some time.

The latter was separated with difficulty. In two cases a third type of precipitate was noticed, a light brown granular substance that settled out after formation of the waxy and before that of the white material. The precipitates were removed for purification of the cuorin. The alcohol-ether solutions contained the lecithin and some cuorin. Separation of these is discussed later.

Some interesting things were noticed during the next process, that of purification of the cuorin. It was found that when attempts were made to dissolve the alcohol precipitates in cold ether an insoluble material was left. The ether solutions were never quite clear. With some portions a separation was made by centrifuging. This gave good results if carried on for no more than ten minutes. If the supernatant liquid was poured off and re-centrifuged, a copious yellow-white precipitate was secured after five or six minutes. On warming the tubes this substance dissolved but precipitated out when centrifuged. Similar experiences were met with when other portions of extract were treated in a similar fashion. In one extract all the material was thrown out of solution after fifteen minutes centrifuging.

On account of these results and the danger of loss of substance the separation by centrifuging was discontinued. The alcoholic precipitates were treated with cold ether, then warmed until clear solutions were secured. These were now set in cylinders in the refrigerator. On standing, the insoluble materials crystallized out. Though this insoluble substance seemed to be present in large amounts, only a little dried and purified

material was secured.

A nitrogen analysis gave the following--

.5395 g required 1.68 cc. N/10 H_2SO_4

%N in sample = .436.

This low value seems to show that a fat-like substance had passed through the various treatments.

The clear ether solutions were precipitated by absolute alcohol. Purification by solution and reprecipitation was continued until no ether insoluble material was found. The final preparation of cuorin was made by pouring the clear dark red ether solutions into alcohol, instead of adding the alcohol to the ether. By this method an excess of alcohol was always present, thus preventing the formation of a precipitate of the waxy type. The brown granular cuorin settles out within a few minutes. It was filtered and dried.

This cuorin after drying was a light brown granular substance. It was found that cuorin secured in this way was practically insoluble in ether. It is uncertain whether this is due to strong dehydration changing the physical properties or whether the alcohol affects the chemical nature of the substance.

For this work it was not considered necessary to have the substances absolutely free from impurities. Cuorin and lecithin sufficiently pure to give comparable hydrolysis products were required. The cuorin was analyzed as follows:

Nitrogen

A .4810 g required 3.71 cc. N/10 H_2SO_4

% N = 1.08

B .3345 g required 2.62 cc. N/10 H_2SO_4

% N = 1.09

The alcohol ether solution from the final precipitation of cuorin was pale yellow. After it had stood for an hour or two, a white flocculent precipitate was noticed in it. The solution was allowed to stand over night, then filtered. The yield of precipitate was very slight, too slight to make analyses that were of any value.

Lecithin was secured by evaporation of the alcohol ether solutions from early precipitations of cuorin. These liquids were clear dark red or brown. Some portions were evaporated at atmospheric pressure, others in a partial vacuum. The concentrates were taken up in absolute alcohol when some material remained undissolved. The alcoholic solution was filtered and evaporated. The concentrate was taken up in anhydrous ether and finally precipitated with anhydrous acetone.

Lecithin is a dark brown waxy mass, easily soluble in ether and alcohol. No weights were taken on the separated phosphatides but observations seemed to show that there is four to five times as much lecithin as cuorin in heart muscle.

Analysis of lecithin for approximation of purity,

Nitrogen

.4887 g required 6.87 cc. N/10 H_2SO_4

% N in sample = 1.96

B. Hydrolysis.

The determination of the structure of substances by studying their cleavage products is a process much used in organic chemistry. Where good separations and sufficient yields are secured, this system is of great value. It has already been shown how by means of hydrolysis products a tentative formula was developed for lecithin and how by the same means this formula was proved incorrect.

For hydrolysis of phosphatides dilute aqueous solutions of $Ba(OH)_2$, H_2SO_4 and HCl, methyl alcoholic solution of $Ba(OH)_2$ and solution of HCl in ethyl alcohol have been used. A danger reported in the use of H_2SO_4 is that oxidation of the unstable substances is made more easy. There have also been objections raised against the use of $Ba(OH)_2$ on account of possible liberation of NH_3. Dilute HCl had previously served very satisfactorily in this laboratory as a hydrolizing agent so its use was continued throughout these experiments.

(1) General Plan. For every gram of phosphatide 100 cc. 10% HCl (10 cc. con. HCl:90 cc. H_2O) were used. The material and acid were introduced into a Kjeldahl flask and boiled gently for 19-25 hours, using a return condenser.

The visible result of this treatment is a separation of the compound into the insoluble fatty acid residue floating on top of the liquid and into the soluble portions forming a yellow or brown solution, as the case might be, with the acid.

As this was a study of nitrogen distribution and separation of the nitrogen containing compounds, the fatty acids

and glycerophosphoric acid were not taken up. The nitrogen containing portions were separated into five groups for quantitative determinations as outlined in the following paragraphs.

After hydrolysis was considered completed, the solution was filtered off and the flask and residue washed with 5% HCl, which was added to the filtrate. The filter paper containing the residue was returned to the flask for a determination of the nitrogen in the fatty acid fraction.

The filtrate was evaporated on the steam bath. More fatty acids separated out on concentration and were added to the residue. When the concentrate had reached a syrupy consistency, it was taken up in absolute alcohol. Solubility in alcohol seems to depend somewhat on the extent of the previous concentration. If evaporation is carried too far there is a rather large amount of insoluble material and some of the nitrogen containing substance may be held in it. If the treatment goes no farther than the liquid stage the salts and glycerophosphoric acid which are insoluble in absolute alcohol may be taken up somewhat by the more dilute reagent. Nitrogen determinations were made on the residue, called hereafter the alcohol insoluble residue.

The filtrate from the alcoholic treatment was concentrated and alcoholic H_2PtCl_6 added as long as a precipitate was formed. This precipitate consists of the platinum compound of the nitrogenous bases or the di-amino acids split off during hydrolysis. The substance was yellow or orange-yellow and in the first stage possessed no crystalline form.

The liquid was filtered off from the platinum precipitate, diluted, warmed and treated with H_2S to remove excess

Pt. Dilution and warming are necessary to bring about dissociation of the compound so that the Pt. may be precipitated.

The next step is the separation from the filtrate of the substance corresponding to a mono-amino acid. The filtrate was made alkaline by addition of a few drops of saturated Na_2CO_3. A saturated solution of $Hg(C_2H_3O_2)_2$ was then added drop by drop as long as a white precipitate formed. The appearance of a yellow precipitate marked the end of the reaction. Nitrogen determination gave the amount present in the mono-amino acid fraction.

The filtrate from the $Hg(C_2H_3O_2)_2$ precipitation always contains some nitrogenous compound along with the other substances. It is not known what this corresponds to in the phosphatide molecule.

(2) Hydrolysis of Cuorin. This involved two treatments, the quantitative determination outlined above for the study of the nitrogen distribution and a qualitative one for separation of the nitrogen containing substances.

For the distribution one hydrolysis was made. This was considered sufficient, for the nitrogen total of the products checked with that secured on analysis of the unhydrolized cuorin. 1.1988 g. were hydrolized for 21 hours. The fatty acid residue was black and sticky while the substance itself before hydrolysis was brown and more granular in character. The process apparently has an oxidizing effect on the acids themselves, as well as separating them. The filtrate was a clear yellow-brown liquid. On concentration and treatment with absolute alcohol a gray substance with the consistency of putty remained

insoluble. Extractions with hot alcohol seemed to have no effect on this substance. The alcoholic extracts were united, concentrated and treated with H_2PtCl_6. A yellow amorphous precipitate formed. This was not very soluble in cold water but readily went into solution on heating. Addition of warm absolute alcohol to this solution to turbidity caused the formation of yellow octahedral crystals on cooling. Separation of the mono-amino-acid fraction, as described above, gave a bulky precipitate. The yellow filtrate from this treatment was concentrated and analyzed for nitrogen.

Percentages of Nitrogen

(A) in sample; (B) as portion of total N.

Fraction	A	B
Fatty acid residue	.183	17.
Alcohol insoluble residue	.101	9.50
Platinum chloride precipitate	.163	15.19
Mercuric acetate precipitate	.507	47.25
Filtrate	.119	11.09
Totals.....	1.073	100.03

Discussion of these figures will be given later.

In the hydrolysis for the separation of the products 6.33 g cuorin were hydrolized for 25 hours. The general method of separation was carried through as above.

Descriptions of the fractions show:

Fatty Acid Residue--was a black sticky mass, firmly

solidified at room temperature. It was set aside for future treatment.

Alcohol Insoluble Residue--After two evaporations of the hydrolysis filtrate the concentrated liquid was treated with alcohol. A division of the insoluble portions was observed. Besides the substances usually noticed, a gray waxy compound was present. On treatment with hot water much of it dissolved along with the salts. It was probably glycerophosphoric acid set free during hydrolysis.

The residue was dried and treated in several ways. A small amount of the solid was tested for NH_3 with $Ca(OH)_2$ and H_2O. Result, positive. The granular portion was not completely soluble in water but as the insoluble portion contained no nitrogen it was not kept. The solution was clear and colorless. On evaporation and crystallization several types of crystals were noticed. A portion of the solution tested with Nessler's reagent gave good positive results. When the solution was treated with $H_2(Pt)Cl_6$ a yellow amorphous precipitate was secured. On further treatment this was found to consist of regular octahedra. This is the characteristic crystal form of NH_4Cl and KCl compounds with Pt. In connection with the other tests the presence of NH_3 as a hydrolysis product is thus strongly indicated.

Platinum Chloride Compound--The precipitate was composed of orange yellow crystals and some amorphous substance. The crystals were not very soluble in cold H_2O but dissolved readily on heating the liquid. When re-crystallized either from water or from alcohol-water mixture they formed yellow truncated octahedra. A uniform type was secured by repeated crys-

tallization, but the amount of substance was small. At one stage of purification the addition of warm alcohol to the water solution caused a dissociation of the Pt compound on standing, with liberation of the metal. This experience had previously been met with in this laboratory with compounds of the type of choline. The crystal form of the substance also corresponds to that of members of the choline group.

Mercuric Acetate Precipitate--This precipitate is a bulky yellow-white mass having a peculiar odor. It was finely powdered, suspended in hot water, the liquid acidified slightly with HCl and treated for several hours with H_2S. This breaks up the Hg compound, removing the metal as the sulphide. The HgS was filtered off, ground up, suspended, and treated again with H_2S. After this treatment the two filtrates from the HgS were filtered and evaporated almost to dryness. Salts such as NaCl and Na_2CO_3 are here separated from the organic compounds when alcohol is added. On the second crystallization from alcohol fine white leaf-like crystals have formed. An attempt will be made to purify and characterize this substance.

Mercuric Filtrate--This yellow liquid was evaporated completely to dryness. A yellow fat-like substance remained as residue. A scheme for the investigation of this is to suspend it in water, add AgOH and study the organic Ag compound thus secured.

(3) Hydrolysis of Lecithin. The procedure was the same as for cuorin. The fatty acids and the nitrogen values differed from those of cuorin. Otherwise the same general characteristics of the products were noted. 1.0414 g of lecithin

gave the following nitrogen distribution.

Percentage of Nitrogen

(A) as in sample; (B) in terms of total N.

Fraction	A	B
Fatty acid residue	.231	11.91
Alcohol insoluble residue	.103	5.31
Platinum chloride precipitate	.808	41.65
Mercuric acetate precipitate	.674	34.74
Filtrate	.124	6.37
Totals.....	1.940	99.98

Another sample gave a determination for nitrogen in the Pt. fraction corresponding to 42.51% in a sample whose total nitrogen content was 2.016%.

For the separation of the hydrolysis products 21.46 g lecithin were hydrolized for 25 hours. After this treatment the system of separation used with cuorin was carried out.

Fatty Acid Residue--This seemed to consist of two substances, as a separation by means of relative color was easy to determine in the flask. No such distinction could be noted later however. The acids are not firmly solidified at room temperature and for this reason a difficulty was encountered in the first filtration. When this process was carried on in the ice box, it was slow but the filtrate was clear. On concentration of it a brown leathery substance settled out. This was insoluble in the 5% HCl used for rinsing out the flasks, insoluble in ether, but soluble in absolute alcohol giving a clear

dark brown solution. A qualitative test for N was negative.

Alcohol Insoluble Residue--This was more granular and less bulky than that of cuorin. It is not completely soluble in water. Tests of the aqueous solution of the residue by Nessler's reagent and by boiling with $Ca(OH)_2$ were positive for NH_3.

Basic Nitrogen Fraction--On account of the rather large amount of nitrogenous bases present in the alcohol solution, it was thought best to use a saturated alcoholic solution of $HgCl_2$ instead of H_2PtCl_6 as the precipitating agent. At first 40 cc. $HgCl_2$ were added, forming a yellow precipitate. On purification this precipitate was found to be almost white. Tests seemed to show that an excess of $HgCl_2$ was present in the solution, so it was warmed and treated with H_2S. No precipitate of HgS appeared, even on dilution, until after the formation of a grayish white and a yellow substance. It was thought that perhaps not enough $HgCl_2$ was present so the precipitates were filtered off and more $HgCl_2$ added. Another precipitate somewhat similar to the first one was thrown down. After standing it was filtered off and set aside for comparison. Treatment with H_2S of the filtrate from this reaction gave a repetition of the previous results, the gray precipitate formed at first, followed after some time by the black one of HgS. The H_2S precipitates when thoroughly washed gave no good qualitative tests for N, though traces were observed.

The precipitation with $HgCl_2$ gives a compound which agrees in crystalline form with that of choline. No other means of identification was tried. The action was not complete for

after removing the excess of Hg from the alcoholic solution a measurable quantity of precipitate was secured with H_2PtCl_6. This was recrystallized and examined. It was seen to be similar to the combination of platinum and choline.

The separation of the amino acid fraction and the filtrate will be carried out as with cuorin.

(4) Hydrolysis of Ether Insoluble Material. (See page 18). A hydrolysis for the nitrogen distribution in the white substance insoluble in ether was also made. 1.106 g were hydrolized for 21 hours.

Percentages of Nitrogen

(A and B as before)

Fraction	A	B
Fatty acid residue	.675	27.61
Alcohol insoluble residue	.371	15.18
Platinum chloride precipitate	.372	15.22
Mercuric acetate precipitate	.895	36.62
Filtrate	.131	5.36
Totals.....	2.444	99.99

The fatty acid residue was pale yellow, did not seem to have undergone any such change as took place with cuorin and lecithin. The alcohol insoluble residue consisted almost altogether of granular material, salts which had not previously been removed from the substance. The H_2PtCl_4 precipitate was of no definite crystalline form in the stage at which it was examined. It seemed to be lighter in color than Pt. compounds of

the other substances and more soluble in H_2O. The $Hg(C_2H_3O_2)_2$ precipitate was similar to that of other phosphatides. The filtrate was almost colorless.

IV . DISCUSSION.

For dehydration of tissue, acetone seems to be the best medium although its application in this series of experiments was not extensive. In the alcohol treatment two results, undesirable for this work, are possible. The effect most to be avoided is that of the action of the reagent in dissolving some of the phosphatides and transferring them to the alcohol-water mixture. Also the solubility of these substances in the ether used later is perhaps altered. The statement by Erlandsen that there is danger of oxidation in dehydration by alcohol would apply only in the study of the more unstable substances in the molecule, the unsaturated fatty acids. No change was observed that would interfere with the radicals investigated. The preparation of tissue by air drying also has some disadvantages. The difficulty reported by MacLean in securing pure phosphatides shows how far the nitrogenous impurities continue through the steps of the preparation of lecithin. He used tissue dried in a current of warm air. Certainly it would seem that any dehydrating agent which is thorough in its action, takes up a good proportion of nitrogenous substances which might interfere later, does not dissolve phosphatides, and leaves the tissue in good condition for extraction is the best agent to use.

Acetone dehydration, as it is now practiced in this laboratory, fulfills these specifications.

The extraction of the substances from the tissue is a problem in itself. The results secured bear out the statements that the phosphatides are not found free in the tissues but are in some combination. This combination is probably of two types, labile and stabile. The former is broken up completely by the extraction in the Soxhlet apparatus. The latter requires the more vigorous treatment with boiling alcohol. After the phosphatides are removed the alcohol takes up increased amounts of other substances, probably nitrogenous extractives. These contaminate the preparations to some extent, expecially in air dried tissue.

The cuorin secured by the method of precipitation used, differs by its insolubility in ether after this treatment from that previously reported. It was also noticed that the phosphatide which is insoluble in anhydrous ether is present in slightly larger quantities than has been supposed.

The distribution of the nitrogen in the cuorin, lecithin, and ether insoluble phosphatide points the way for further investigation of the hydrolysis products of each of these substances. Still it is not proved definitely that there are five forms of nitrogen, although the evidence leads in that direction. The nitrogen in the fatty acid residue may be an impurity and the phosphatide itself may be completely hydrolized. The filtrate from the treatment with $Hg(C_2H_3O_2)_2$ may contain some of the mono amino acid fraction for the precipitation is not always quantitative.

The alcohol insoluble residue certainly has NH_3 in it in the case of cuorin and lecithin. It is not definitely known whether this is a primary product of the action or a secondary one due to further breaking up of the compounds secured by the hydrolysis. The NH_3 may be present in an amide. The amount split off is quite constant with kephalin and probably is with the compounds in the heart also.

MacLean at one time thought that perhaps the choline in lecithin was broken up during hydrolysis and volatile cleavage products liberated. His later work, however, showed that he could recover 96% of the original amount of choline after subjecting it to the treatment. By reference to the distribution tables it will be seen that in lecithin the nitrogen in the alcohol insoluble residue amounts to 5% of the total and about 20% of that in the platinum chloride compound (basic). With cuorin the relation is 10% of the total or 60% of the basic nitrogen in the alcoholic residue. These amounts are evidently not explained by decomposition of the nitrogenous base.

The basic compound of cuorin has been separated as the Pt. salt and is now in a rather pure state, ready for further tests. The crystals of the substance look like those of the choline-platinum compound from brain lecithin. The salts of neurine and other bases crystallize in this same system so nothing definite can be told from that. The higher per cent of Pt. reported by Erlandsen may be due to contamination with H_2PtCl_6. Gulewitsch[12] says that often some of the reagent may be carried down in the amorphous precipitate and that it is separated with difficulty.

In curoin the basic nitrogenous substance was found to be present in relatively small proportions, one-sixth of the total nitrogen. In lecithin the amount of choline nitrogen checked with that reported by Erlandsen and MacLean. The total nitrogen per cent in the samples worked with was somewhat higher, however.

Much the larger portion of the nitrogen present in cuorin was found in the amino acid fraction. This, in connection with the statement by Erlandsen that on carrying through tests for fatty acids he secured results that seemed to show the presence of three in the molecule, leads into interesting speculations. The presence of the three fatty acids certainly prevents the adoption of a formula similar to that on page 2, even if all the nitrogen was in one fraction. Previous ideas of the nitrogen containing radicals might perhaps give rise to a formula in which the third fatty acid was connected through the phosphoric acid or the nitrogenous base. The rather general distribution of the nitrogen in the substance dismisses that possibility. The presence of glycerophosphoric acid having been proved, a possible conception is that of connection of two parts of the molecule through the amino acid. This, however, would involve a rearrangement of other radicals so that the nitrogen phosphorous ratio of one to two could be maintained. For the confirmation of any formula advanced the first requisite is the identification of the nitrogen containing radicals. The proportion of fatty acids and glycerophosphoric acid in the molecule would also have to be known.

With the lecithin similar results were obtained. MacLean in the investigation of the hydrolysis of lecithin stated that perhaps an amino acid was present in the molecule, though he had no experimental confirmation of this remark. While such a compound has not as yet been isolated, there is every reason to believe that one is present.

In the ether insoluble phosphatide the nitrogen distribution is somewhat different from that of the others. The large amount in the fatty acid residue may be due to an insoluble product, incomplete hydrolysis or the presence of much undissociable nitrogen, for it is hardly possible that such a large amount of impurity is present. The fatty acids certainly do not resemble those split off from cuorin or lecithin. They are probably more highly saturated. The percent of total nitrogen in the mono amino acid fraction is practically equal to that in lecithin. There may be some connection, though until more is known about the substance it can not be brought out.

The results of the work on the phosphatides of the heart demonstrate that with them as with kephalin the problem of the nitrogen containing radicals is still more complex than previous reports would show. It muxt be repeated that only through the study of these compounds will explanation of general structure of the phosphatides be attained.

At the present time it is equally necessary to work out the chemistry and the physiology of the phosphatides.

V.. SUMMARY.

From the experimental and theoretical considerations the following summary can be made:

1. For preparation of heart tissue for extraction, dehydration by acetone is the most satisfactory method.

2. Phosphatides in the tissues are in two forms of combination, labile and stabile.

3. For cuorin, lecithin and the ether insoluble phosphatide the nitrogen in each,can, with some qualifications, be distributed into five different forms.

4. It is highly probable that ammonia is a hydrolysis product of cuorin and lecithin.

5. The properties of the nitrogenous base in cuorin are similar to those of choline.

6. A substance corresponding to the hydrochloride of an amino acid has been isolated from cuorin. A similar substance is probably present in lecithin and the ether insoluble phosphatide.

7. There is need of further investigation of the phosphatides to correlate their chemical and physiological behavior. Their chemistry will be best understood by study of their nitrogen-containing radicals.

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